AD_____

Award Number:

   W81XWH-11-1-0466

TITLE:

   B7-H4 as a Target for Breast Cancer Immunotherapy

PRINCIPAL INVESTIGATOR:

   Alan L. Epstein MD, PhD

CONTRACTING ORGANIZATION:

   University of Southern California
   Los Angeles, CA 90033

REPORT DATE:

   June 2012

TYPE OF REPORT:

   Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                   Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                   Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-06-2012	Annual	1 Jun 2011 - 31 May 2012

4. TITLE AND SUBTITLE

B7-H4 as a Target for Breast Cancer Immunotherapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0466

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Peisheng Hu, Julie K. Jang, and Alan L. Epstein

E-Mail: aepstein@usc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Southern California

Los Angeles, CA 90033

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

B7-H4 is a recently discovered B7-family molecule that has been shown to inhibit T cell proliferation and secretion of IL-2. Therefore, it has been classified as an immunosuppressive protein. Protein expression has been limited to subsets of activated T cells and is inducible in dendritic cells and macrophages. In contrast, protein expression is abundant on tissues from several malignancies, most notably breast adenocarcinoma. We proposed to generate antagonistic humanized anti-B7-H4 antibodies for the reversal of immune escape generated in breast cancer. Here we report the generation of 64 mouse monoclonal antibodies for the detection of B7-H4 by ELISA, and 25 for the detection of cell surface B7-H4 by flow cytometry. We are currently assessing the 25 antibodies suitable for flow cytometry for direct cytotoxic effect on human breast cancer cell lines as well as for antagonistic effects on B7-H4 function. Candidate antibodies will be subsequently humanized using genetic engineering techniques. Here, we also report several novel findings not yet reported in published literature and not anticipated in our grant proposal.

15. SUBJECT TERMS
B7-H4, immunotherapy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	18	**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U			

Table of Contents

Introduction

In order for tumors to survive, the cancer must avoid elimination by the host's immune system either by avoiding detection or by inducing suppressive mechanisms. Inhibition of the immune response in the tumor microenvironment can be accomplished by the secretion of immunosuppressive cytokines, like TGF-β, the recruitment of suppressor cells such as T regulatory cells and myeloid derived suppressor cells (MDSC), or the expression of immunomodulatory molecules at the cell surface like CTLA-4, PD-1, and B7-H4. B7-H4, the most recently discovered of the B7-like molecules, is not as well characterized as other cell surface suppressor molecules. Its mRNA expression is ubiquitous but its protein expression has been limited to subsets of activated T cells and antigen presenting cells [1]. Many human cancers also express B7-H4 such as renal cell carcinoma [2], melanoma [3], prostate cancer [4], ovarian cancer [5], and breast cancer [6,7]. Recent characterization of the molecule has shown that B7-H4 can inhibit T cell proliferation and IL-2 secretion [8], and thus, it has been hypothesized to protect tumor cells from T cell-mediated destruction. We proposed the development of a humanized antagonistic antibody to B7-H4 that could be used to treat B7-H4 expressing tumors such as breast cancer as a new immunotherapeutic approach for cancer patients.

Body

Specific Aim 1. Generation of an antagonistic humanized B7-H4 antibody.

Aside from characterization of B7-H4 expression in tumor tissues, almost all work with B7-H4 has been done in murine models. For the purpose of our studies with human tissues, we developed a human B7-H4-Fc fusion protein that consists of the extracellular domains of human B7-H4 fused to the Fc region of human IgG1 **(Fig. 1)**. In concurrence with published data in mice [8], human B7-H4-Fc was able to bind to human T cells activated with anti-CD3/CD28 beads, as well as on some dendritic cells, macrophages, and B-cells **(Fig. 2)**, as detected by flow cytometry. Interestingly, *in vitro* activation of T cells, but not of the other cell populations, was required for expression of the B7-H4 receptor.

Using APC-labeled B7-H4-Fc, we have discovered B7-H4 receptors on several human lymphoma and leukemia cell lines. CEM, Karpas 299, and TLBR-1, cell lines derived from acute T-cell lymphoblastic leukemia, large cell anaplastic lymphoma, and breast implant-associated anaplastic large cell lymphoma, respectively, bound B7-H4-Fc, but Jurkat, an acute T-cell leukemia-derived cell line, did not bind B7-H4-Fc (Fig. 3). This novel finding was unanticipated and exciting, as it open new doors to the study and treatment of hematopoietic malignancies. If engagement of the B7-H4 receptor with B7-H4 is able to inhibit lymphocytic proliferation, B7-H4-Fc may be able to be administered as treatment against malignant lymphocytes. Furthermore, B7-H4-Fc can also be used as a targeting molecule to deliver cytotoxic drugs to these malignant cells. This work on T-cell lymphomas was motivated by our need to find good B7-H4 receptor expressing cell lines that could be used in subsequent experiments.

In anticipation of developing antagonistic antibodies to B7-H4, we developed an assay to test the suppressive effects of B7-H4 on T cell proliferation isolated from peripheral blood of health donors. Surprisingly, however, our data could not reproduce the *in vitro* suppression achieved with murine B7-H4 on murine T cells [8]. Several different methods of presenting B7-H4-Fc to T cells were tested, including using soluble B7-H4-Fc, and immobilized B7-H4-Fc attached to the surface of 96-well plates, Dynabeads (Invitrogen), or copolymer beads. We also tried different ways of stimulating T cells, including anti-CD3 (clone HIT3a) and anti-CD3/CD28 Dynabeads, both of which stimulated T cell proliferation. In addition to different concentrations of B7-H4, different time points of collection and different readouts, including proliferation measured by CFSE and the presence of activation markers like CD25 and CD69, were tested. In each experiment, T cell proliferation and activation did not differ significantly between B7-H4-treated and IgG1-treated cells (Fig. 4).

Several possibilities may explain the discordance between our findings with human B7-H4 and published work using murine B7-H4. It may be that B7-H4 is an evolutionarily conserved molecule but whose function and signaling may be redundant or easily surmounted in humans versus mice. It may also be that B7-H4 may need to cluster, oligomerize, or be presented by viable cells in order to convey suppressive

signaling. To elucidate the role of B7-H4 in the human immune system, we shifted our studies to the identification of the B7-H4 receptor on activated T cells. Identification of the receptor may provide important information about the function of B7-H4 in human tumors. It is also possible that antagonistic antibodies may bind either B7-H4 alone, its receptor, or the ligand/receptor complex so information about the molecular identity of the receptor is vital for these studies. We have already begun performing pull-down assays using B7-H4-Fc as bait. Initial studies performed by our laboratory have shown that the receptor is protease insensitive as suggested by digestion and flow cytometry experiments (Fig. 5). Based upon these results, it is possible that the receptor is not a protein and other techniques need to be used to identify its molecular composition.

Even though *in vitro* studies with B7-H4-Fc did not suppress T cell proliferation, our goal has not changed to produce an antagonist human antibody. The abundance of B7-H4 on the surface of over 95% of breast cancer cases [6], lack of protein expression in normal tissues, and its reverse correlation with prognosis [3,4,5] make B7-H4 an excellent target for antibody-mediated or directed therapy. In addition, intracellular B7-H4 has been shown to have anti-apoptotic effects in biliary epithelial cells from patients with primary biliary cirrhosis [9]. In biliary epithelial cells acquired from primary biliary cirrhosis, Chen *et al*. [9] showed that silencing of B7-H4 using RNA interference was able to induce apoptosis in those cells without the addition of other cells, like cytotoxic T lymphocytes, or cytotoxic molecules. It is conceivable that B7-H4 conveys protective effects in tumor cells apart from its immunomodulatory effects. An antagonist antibody can still be made to inhibit its other functions.

In preparation of producing a human or humanized antibody, studies were undertaken to produce murine hybridomas that may be used to define functional assays. For these studies, monoclonal antibodies against B7-H4 have been generated by immunizing BALB/c and NIH Swiss mice with B7-H4-Fc in Freund's adjuvant and other proprietary immune activators. After 3-5 rounds of immunizations, including a final boost 4 days before sacrificing the mice, splenocytes from immunized mice were fused with mouse myeloma NS-0 cells and culture supernatants were screened for binding to B7-H4-Fc by ELISA. Positive supernatants were then screened against other Fc fusion

molecules generated in the laboratory (IL-2-Fc, B7.1-Fc) to identify antibodies that bound to the Fc portion of B7-H4-Fc. A total of 64 antibodies have been generated that recognizes the extracellular domain of B7-H4-Fc by ELISA. Those antibodies were then screened by flow cytometry against the B7-H4 positive human breast adenocarcinoma cell line SKBR-3. Twenty-five of the 64 antibodies were found to bind and are therefore suitable for use by flow cytometry (Fig. 6). By comparison, a commercial anti-B7-H4 antibody (H74, eBiosciences) was not able to bind SKBR-3, possibly due to its lower affinity or to the epitope that it binds.

Specific Aim 2. Evaluation of the clinical efficacy of B7-H4 antibodies in 4T1 murine breast cancer model and in human breast tumor-bearing mice.

We have begun to screen for direct cytotoxic effects of the newly generated murine antibodies against B7-H4 positive (SKBR-3) and negative (JAR, HT29, and T47D) cell lines. We already have several promising antibodies yielding greater rates of apoptosis than HER-2 antibodies in SKBR3 (Fig. 7). Humanization of these candidate antibodies will allow us to study these antibodies for antibody-dependent cell cytotoxicity and further study their therapeutic effects in SKBR-3 xenograft tumor models in mice.

We are currently assessing the immunosuppressive effects of human B7-H4-Fc on stimulated murine T cells *in vitro*. Because of the high homology between mouse and human B7-H4 (87%), there may be cross-species receptor binding. Furthermore, we will be screening the mouse monoclonal anti-human B7-H4 antibodies for reactivity to mouse B7-H4 by flow cytometry and for apoptotic activity. Those candidate antibodies will be used for studies in an *in vivo* 4T1 murine breast cancer model in BALB/c mice.

Specific Aim3. Analyze human breast cancer biopsies for the expression of B7-H4 and tumor infiltrating lymphocytes.

Breast cancer specimens from the USC Norris Cancer Center have been requested from Mr. Terry Church who is the new Director of the Breast Cancer Program at the USC Keck School of Medicine. We have already titrated the following antibodies for immunohistochemistry of paraffin-embedded sections: CD3, CD8, CD4, FoxP3, CD25, MHC class II, CD19, CD11c, and CD68.

Key Research Accomplishments

- Generation of human B7-H4-Fc fusion protein (antigen).
- Discovery of a B7-H4 receptor on CEM, Karpas 299, and TLBR-1 cell lines derived from T-cell acute lymphoblastic leukemia, large cell anaplastic lymphoma, and breast implant-associated anaplastic large cell lymphoma, respectively.
- Generation of 64 mouse anti-human B7-H4 monoclonal antibodies for use in the detection of B7-H4 by ELISA.
- Of the 64 monoclonal antibodies suitable for ELISA, 25 mouse anti-human B7-H4 monoclonal antibodies can detect cell surface B7-H4 by flow cytometry.
- Tested direct cytotoxicity of candidate B7-H4 antibodies.

Reportable Outcomes

- Training grant application in review for the NIH Training Program at USC in Cellular, Biochemical, and Molecular Sciences.

Conclusion

We have generated a large selection of mouse anti-human B7-H4 monoclonal antibodies that we can now assess for antagonistic activity, antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity, and direct cytotoxicity. Humanization of the antibodies with the highest activities will be performed in order to develop clinically applicable antibodies. More work still needs to be done characterizing the role of B7-H4 in the human setting. However, regardless of B7-H4's precise role in the immune response, the expression pattern of B7-H4 and its correlation with prognosis make B7-H4 an excellent target for antibody-mediated or directed therapy in breast cancer. After confirming some of our *in vitro* results, we will be ready for humanization of the antibodies and subsequent testing *in vitro* and *in vivo*. If the receptor for B7-H4 is identified, this would be a major milestone in the field since prior attempts by many groups have failed. It would also open the door to making a human antibody to the B7-

H4 receptor which would represent another approach for inhibiting this ligand/receptor interaction on breast cancer cells.

We also report here novel findings not anticipated by our original grant proposal. We have demonstrated the presence of the B7-H4 receptor on several lymphoma and leukemia cell lines, as well as its absence in one leukemia cell line. This may clarify some of the biology behind the different phenotypes and behavior of different hematopoietic malignancies. Our work to identify the B7-H4 receptor could shed light on B7-H4's mysterious roles in tumorigenesis and immunology.

References

[1] K.H. Yi, L. Chen, Fine tuning the immune response through B7-H3 and B7-H4. *Immunologic Reviews* **229**, 145-151 (2009).

[2] R.H. Thompson, X. Zang, C.M. Lohse et al., Serum soluble B7x is elevated in renal cell carcinoma patients and is associated with advanced stage. *Cancer Research* **68**, 6054-6058 (2008).

[3] D. Quandt, E. Fiedler, D. Boettcher, W. Ch. Marsch, B. Seliger, B7-H4 expression in human melanoma: its association with patients' survival and antitumor immune response. *Clinical Cancer Research* **17**, 3100-3111 (2011).

[4] X. Zhang, R.H. Thompson, H.A. Al-Ahmadie et al. B7-H3 and B7x are highly expressed in human prostate cancer and associated with disease spread and poor outcome. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 19458-19463 (2007).

[5] I. Simon, D. Iatsaros, I. Rigault de la Longrais et al. B7-H4 is over-expressed in early stage ovarian cancer and is independent of CA125 expression. *Gynecologic Oncology* **106**, 334-341 (2007).

[6] B. Tringler, S. Zhuo, G. Pilkington et al. B7-H4 is highly expressed in ductal and lobular breast cancer. *Clinical Cancer Research* **11**, 1842-1848 (2005).

[7] S. Salceda, T. Tang, M. Kmet et al., The immunomodulatory protein B7-H4 is overexpressed in breast and ovarian cancers and promotes epithelial cell transformations. *Experimental Cell Research* **306**, 128-141 (2005).

[8] G.L. Sica, I.H. Choi, G. Zhu et al. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* **18**, 849-861 (2003).

[9] Y. Chen, G. Guo, S. Guo et al. Intracellular B7-H4 suppresses bile duct epithelial cell apoptosis in human biliary cirrhosis. *Inflammation* **34**, 688-697 (2011).
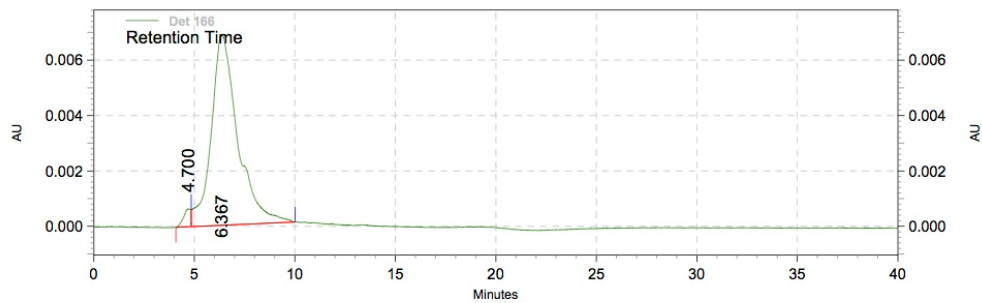
Supporting Data
(Figures)

Figure 1. **Generation of human B7-H4-Fc**. cDNA encoding the signal and extracellular domains of human B7H4 was generated by PCR amplification from full-length cDNA purchased from Open Biosystem (Lafayette, CO). Primary PCR of B7H4 was performed with the 5' and 3' primers 5'-TCG ATC AAG CTT GCC GCC ACC ATG GCT TCC CTG GGG CAG ATC-3' AND 5'-TGT GTG AGT TTT GTC AGC CTT TGA CAG CTG-3', respectively. The hinge-CH2-CH3 portion of human IgG1 was PCR amplified with 5' primer 5'-CTA AAC TCA AGG CTG ACA AAA CTC AC ACA TGC CCA-3' and 3' primer 5'-TGA TTA ATG ATC AAT GAA TTC TCA TTT ACC CGG AGA CAG GGA-3'. The gene encoding huB7H4-Fc was produced by assembling with 5' primer of B7H4 and 3' primer of human, respectively. The B7H4-Fc fusion gene was then digested with *Hind3* and *EcoRI* and inserted into *Hind3* and *EcoRI* sites of pN24 expression vector, resulting in the expression vector pN24/B7H4-Fc. B7H4-Fc fusion protein was expressed in NS0 cell line. Shown is the HPLC profile of B7-H4-Fc.
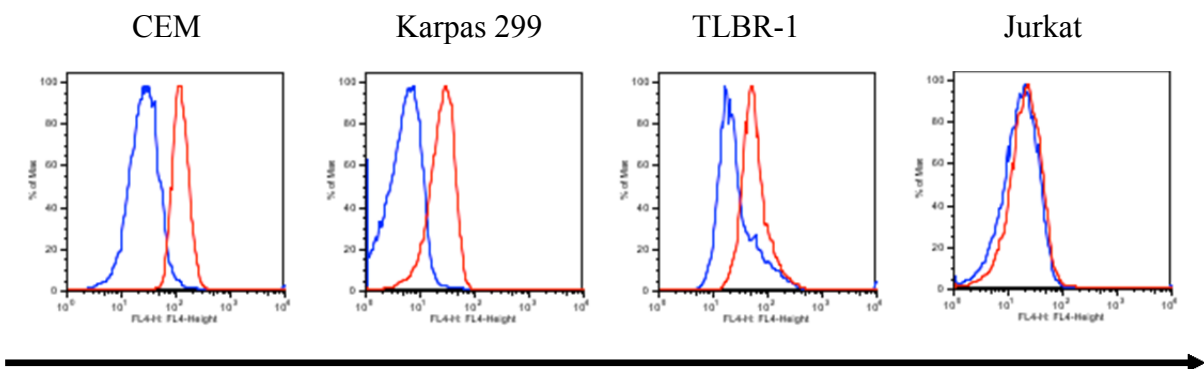
**A**
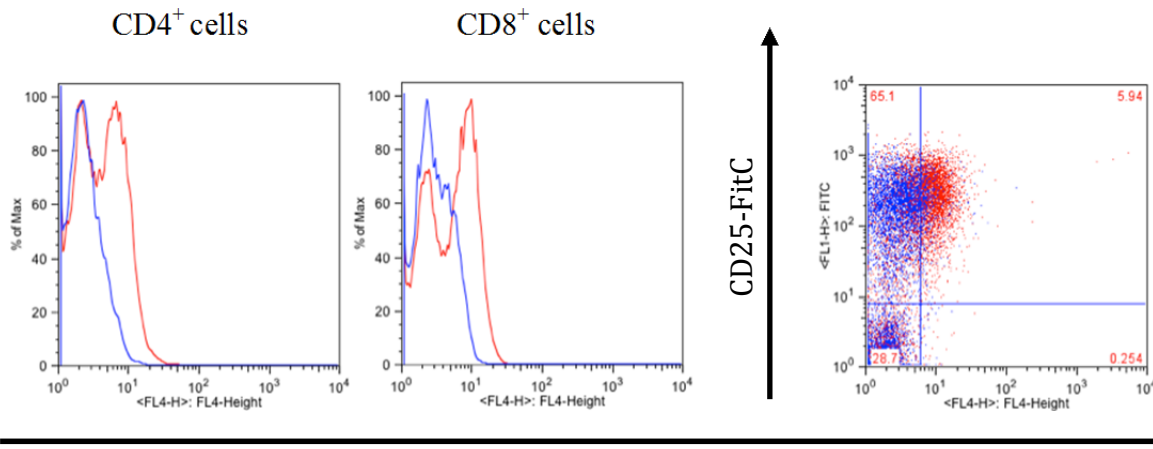
B7-H4R

B
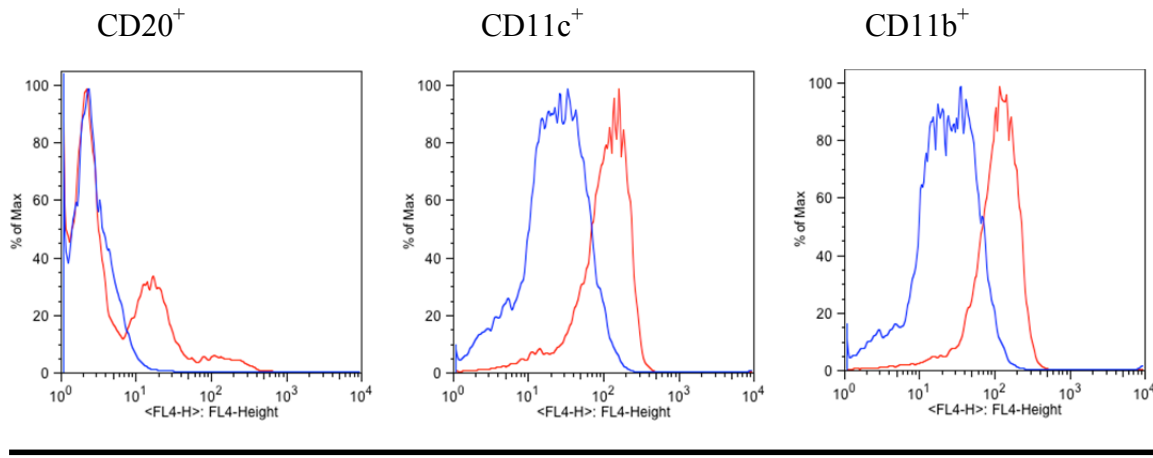
B7-H4R

Figure 2. **B7-H4 receptor positivity on human lymphoid cells**. Red line represents cells stained with B7-H4-Fc-APC, and blue represents cells stained with IgG1-APC. **(A)** $CD3^+$ T cells were isolated from healthy donor peripheral blood and incubated with anti-CD3/CD28 Dynabeads (Invitrogen) for 3 days. Cells were stained with B7-H4-Fc-APC or IgG1-APC, CD4-PE, CD8-PE, and CD25-FITC and analyzed by flow cytometry. Note that CD25 negative cells are also negative for B7-H4R. Non-stimulated T cells did not bind B7-H4-Fc-APC (data not shown). **(B)** $CD3^-$ cells were isolated from healthy donor peripheral blood and incubated with GM-CSF or GM-CSF and Hiltonol (poly-IC:LC) for 3 days. Shown are cells incubated with GM-CSF only. Note B7-H4R positivity on $CD20^+$ cells (B-cells), $CD11c^+$ cells (dendritic cells), and $CD11b^+$ cells (macrophages). Hiltonol-treated cells were similarly positive for B7-H4R (data not shown).

| CEM | Karpas 299 | TLBR-1 | Jurkat |

B7-H4R

Figure 3. **B7-H4 binding to human T-cell lymphoma cell lines**. Red line represents cells stained with B7-H4-Fc-APC, and blue represents cells stained with IgG1-APC. The above cell lines were analyzed by flow cytometry. Note the positivity in CEM, Karpas 299, and TLBR-1 cell lines.

A   anti-CD3/CD28 Dynabeads, B7-H4Fc/IgG1 plated

B7-H4-Fc-treated   **IgG1-treated**
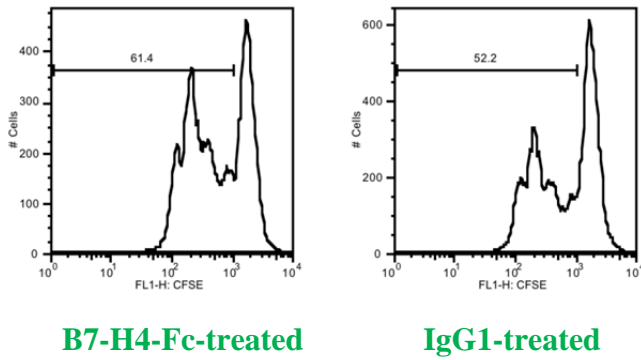
B   anti-CD3 (HIT3a) plated, B7-H4Fc/IgG1 plated

B7-H4-Fc-treated   **IgG1-treated**
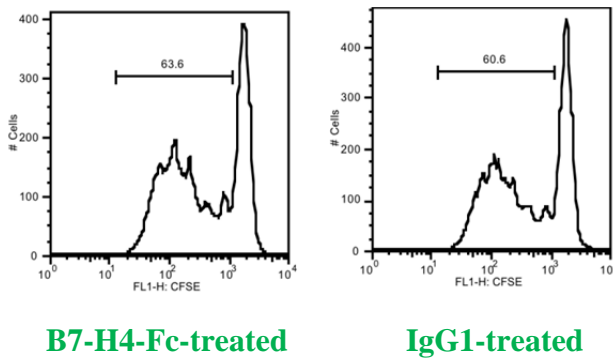
C   anti-CD3 (HIT3a) plated, B7-H4-Fc/FGFR1-Fc soluble

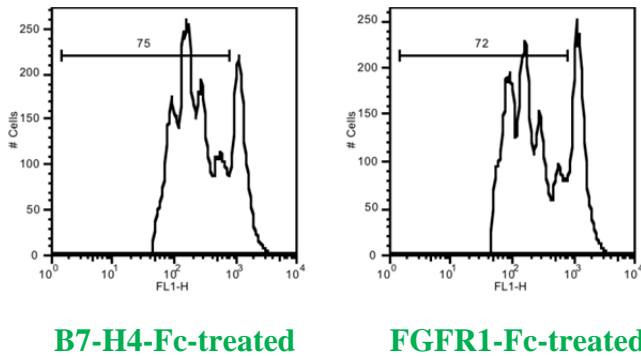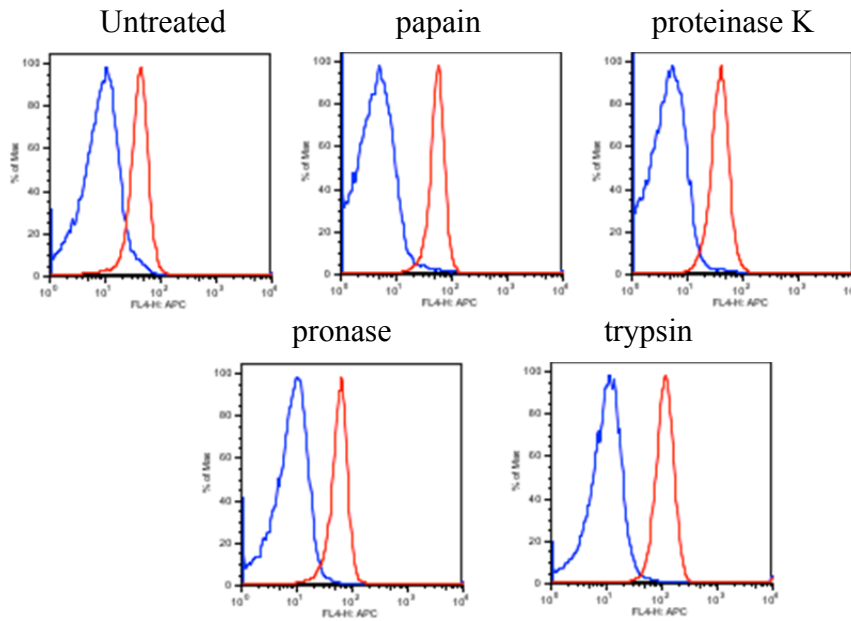**B7-H4-Fc-treated**   **FGFR1-Fc-treated**

Figure 4.   Suppression assay data. (A, B, C) are representatives of several different experiments done trying to demonstrate a suppressive effect of B7-H4-Fc on human T cells. Briefly, in these proliferation experiments, human CD3$^+$ or CD4$^+$ T cells are isolated from healthy donor peripheral blood using magnetic isolation kits from Miltenyi Biotec and stained with CFSE. Cells are treated accordingly and measured for proliferation by flow cytometry. **(A)** Cells were stimulated with anti-CD3/D28 Dynabeads (Invitrogen) and exposed to either B7-H4-Fc or IgG1 plated on 96-well plates for 3 days. **(B)** Cells were stimulated with plated anti-CD3 (clone HIT3a) and exposed to either B7-H4-Fc or IgG1 plated on 96-well plates for 3 days. **(C)** Cells were stimulated with plated anti-CD3 (clone HIT3a) and exposed to soluble B7-H4-Fc or FGFR1-Fc (isotype control).
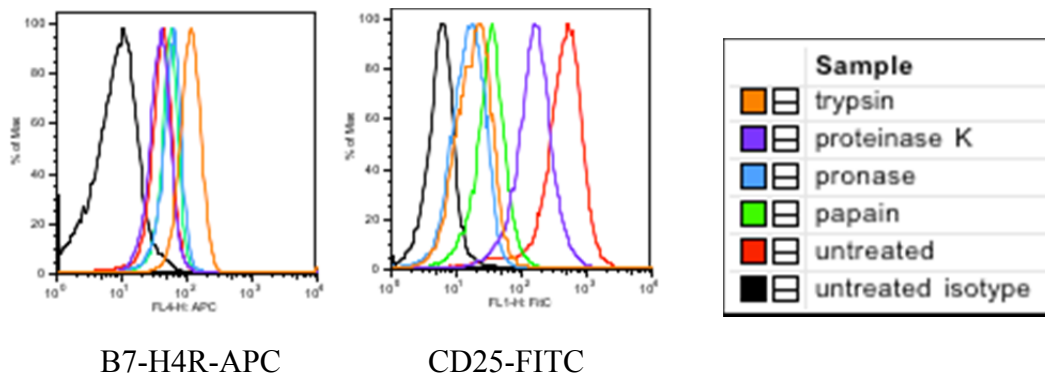
A

Untreated   papain   proteinase K

pronase   trypsin

B7-H4R

B

B7-H4R-APC   CD25-FITC

Sample
trypsin
proteinase K
pronase
papain
untreated
untreated isotype

Figure 5. **B7-H4 receptor is protease resistant.** Karpas 299 **c**ells were treated with 0.3 mg/mL papain, 0.025 mg/mL proteinase K, 0.15 mg/mL pronase, or 0.25% trypsin for 45 minutes at 37°C. Cells were assessed for viability using trypan blue under a light microscope, then stained for B7-H4R using B7-H4-Fc and for CD25 as a positive control for protease activity. (**A**) Flow cytometry data for protease-treated cells for B7-H4R. Red line represents cells stained with B7-H4-Fc-APC, and blue represents cells stained with IgG1-APC. (**B**) Overlays of histogram for B7-H4R and CD25 positivity in protease-treated cells. Note that no protease-treated cells are less positive for B7-H4R than untreated cells (in red), but all protease-treated cells show decreased CD25 expression compared to untreated cells (in red). Therefore, B7-H4R expression as detected by flow cytometry is insensitive to the above proteases, or B7-H4R has a rapid turnover rate and its expression is recovered prior to staining.
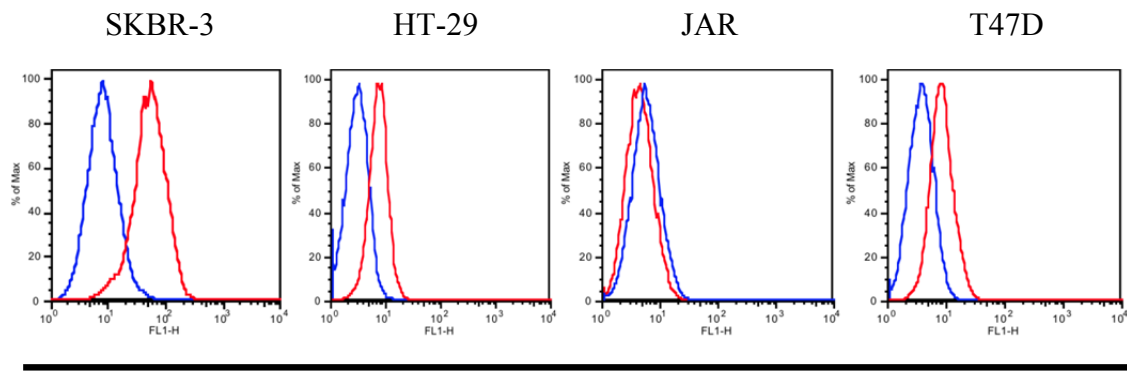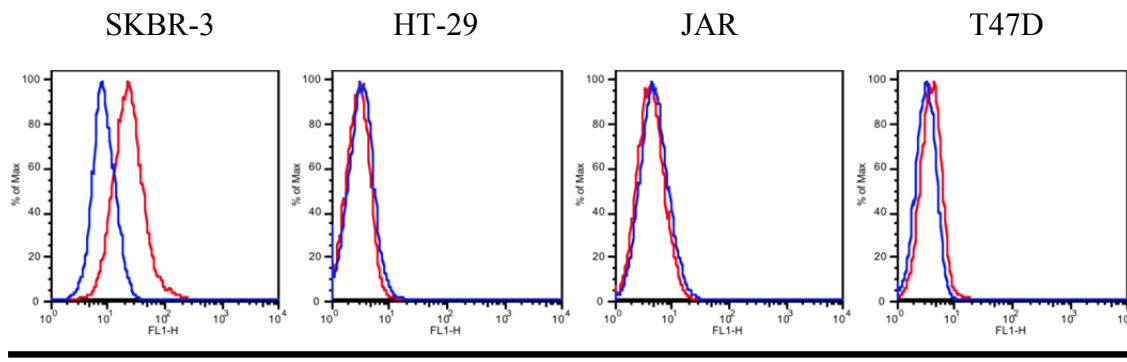
A

SKBR-3   HT-29   JAR   T47D

HER-2

B

SKBR-3   HT-29   JAR   T47D

B7-H4

Figure 6. Representative flow cytometry data for mouse monoclonal anti-human B7-H4 on SKBR-3, HT-29, JAR, and T47D cell lines derived from breast adenocarcinoma, colorectal adenocarcinoma, choriocarcinoma, and breast ductal carcinoma, respectively. Red line represents cells stained for Her-2 or B7-H4, and blue represents cells stained with isotype control. A sheep anti-mouse IgG conjugated to FITC was used as secondary. (**A**) Anti-Her-2 (AHer) was used as a positive control. **B)** Representation of B7-H4 staining. Clone 30 is shown above. Cell surface expression of B7-H4 matches q-PCR data for *b7-h4* expression in these cell lines (data not shown).
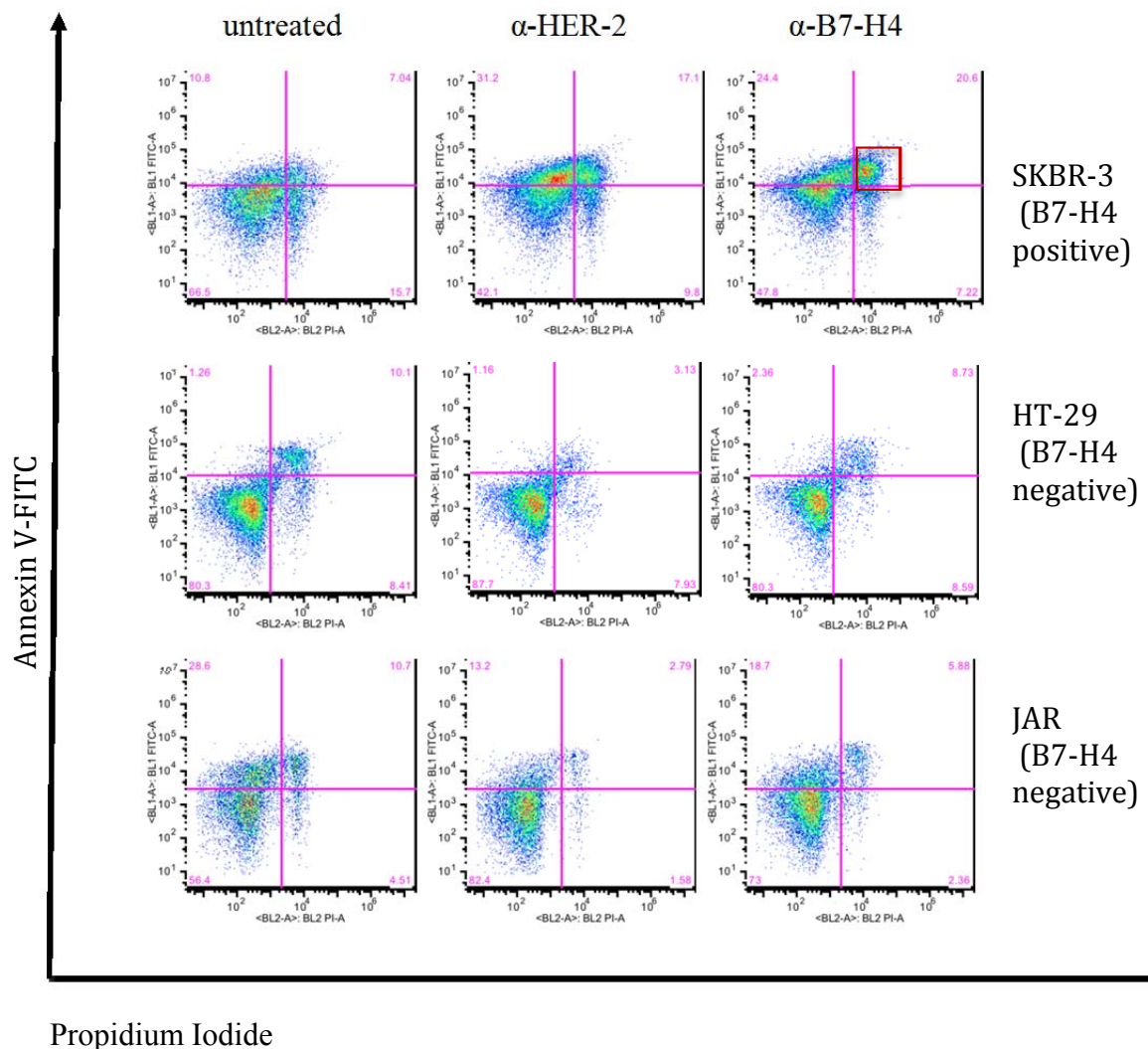
Figure 7. Apoptotic assay for the B7-H4 antibodies. Cells were incubated in the presence of newly generated murine B7-H4 antibodies, HER-2 antibody (AHer) as a positive control, isotype control (not shown), or left untreated for 2 days. Apoptosis was measured using annexin V-FITC and propidium iodide. Apoptotic cells are identified as annexin V positive and propidium iodide negative. Late dead cells are identified as propidium iodide positive. Above is a representation of the data using anti-B7-H4 clone 6D8. Note the slight increase in dead cells in anti-B7-H4-treated SKBR-3 cells compared to anti-HER-2 treated and untreated cells. Twenty different antibodies were screened once in this preliminary experiment.